IMMUNOMODULATORY OLIGONUCLEOTIDES

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Background of the Invention

DNA binds to cell membrane and is internalized

In the 1970's, several investigators reported the binding of high molecular weight DNA to cell membranes (Lerner, R.A., W. Meinke, and D.A. Goldstein. 1971. "Membrane-associated DNA in the cytoplasm of diploid human lymphocytes". *Proc. Natl. Acad. Sci. USA 68:1212*; Agrawal, S.K., R.W. Wagner, P.K. McAllister, and B. Rosenberg. 1975. "Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum-pyrimidine complexes by electron microscopy". *Proc. Natl. Acad. Sci. USA 72:928*). In 1985 Bennett et al. presented the first evidence that DNA binding to lymphocytes is similar to a ligand receptor interaction: binding is saturable, competitive, and leads to DNA endocytosis and degradation (Bennett, R.M., G.T. Gabor, and M.M. Merritt. 1985. "DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA". *J. Clin. Invest. 76:2182*). Like DNA, oligodeoxyribonucleotides (ODNs) are able to enter cells in a saturable, sequence independent, and temperature and energy dependent fashion (reviewed in Jaroszewski, J.W., and J.S. Cohen. 1991. "Cellular uptake of antisense oligodeoxynucleotides". *Advanced Drug Delivery Reviews 6:235*; Akhtar, S., Y. Shoji, and R.L. Juliano. 1992. "Pharmaceutical aspects of the biological stability and membrane transport characteristics of antisense oligonucleotides". In: Gene Regulation: Biology of Antisense RNA and DNA. R.P. Erickson, and J.G. Izant, eds. Raven Press, Ltd. New York, *pp. 133*; and Zhao, Q., T. Waldschmidt, E. Fisher, C.J. Herrera, and A.M. Krieg., 1994. "Stage specific oligonucleotide uptake in murine bone marrow B cell precursors". *Blood*, 84:3660). No receptor for DNA or ODN uptake has yet been cloned, and it is not yet clear whether ODN binding and cell uptake occurs through the same or a different mechanism from that of high molecular weight DNA.

Lymphocyte ODN uptake has been shown to be regulated by cell activation. Spleen cells stimulated with the B cell mitogen LPS had dramatically enhanced ODN uptake

Yano, Y. Kimura, M. Baba, T. Makino, S. Yamamoto, T. Yamamoto, T. Kataoka, and T. Tokunaga. 1992. "Oligonucleotide sequences required for natural killer cell activation". *Jpn. J. Cancer Res. 83:1128*).

Several phosphorothioate modified ODN have been reported to induce in vitro or in vivo B cell stimulation (Tanaka, T., C.C. Chu, and W.E. Paul. 1992. "An antisense oligonucleotide complementary to a sequence in Iγ2b increases γ2b germline transcripts, stimulates B cell DNA synthesis, and inhibits immunoglobulin secretion". *J. Exp. Med. 175:597*; Branda, R.F. , A.L. Moore, L. Mathews, J.J. McCormack, and G. Zon. 1993. "Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1". *Biochem. Pharmacol. 45:2037*; McIntyre, K.W., K. Lombard-Gillooly, J.R. Perez, C. Kunsch, U.M. Sarmiento, J.D. Larigan, K.T. Landreth, and R. Narayanan. 1993. "A sense phosphorothioate oligonucleotide directed to the initiation codon of transcription factor NF-κ β T65 causes sequence-specific immune stimulation". *Antisense Res. Develop. 3:309*; and Pisetsky, D.S., and C.F. Reich. 1993. "Stimulation of murine lymphocyte proliferation by a phosphorothioate oligonucleotide with antisense activity for herpes simplex virus". *Life Sciences 54:101*). These reports do not suggest a common structural motif or sequence element in these ODN that might explain their effects.

The CREB/ATF family of transcription factors and their role in replication

The cAMP response element binding protein (CREB) and activating transcription factor (ATF) or CREB/ATF family of transcription factors is a ubiquitously expressed class of transcription factors of which 11 members have so far been cloned (reviewed in de Groot, R.P., and P. Sassone-Corsi: "Hormonal control of gene expression: Multiplicity and versatility of cyclic adenosine 3',5'-monophosphate-responsive nuclear regulators". *Mol. Endocrin.* 7:145, 1993; Lee, K.A.W., and N. Masson: "Transcriptional regulation by CREB and its relatives". *Biochim. Biophys. Acta* 1174:221, 1993.). They all belong to the basic region/leucine zipper (bZip) class of proteins. All cells appear to express one or more CREB/ATF proteins, but the members expressed and the regulation of mRNA splicing appear to be tissue-specific. Differential splicing of activation domains can determine whether a particular CREB/ATF protein will be a transcriptional inhibitor or activator. Many CREB/ATF proteins activate viral transcription, but some splicing variants which lack the activation domain are inhibitory. CREB/ATF proteins can bind DNA as homo- or hetero- dimers through the cAMP response element, the CRE, the consensus form of which is the unmethylated sequence TGACGTC (binding is abolished if the CpG is methylated) (Iguchi-Ariga, S.M.M., and W. Schaffner: "CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation". *Genes & Develop. 3*:612, 1989.).

The transcriptional activity of the CRE is increased during B cell activation (Xie, H. T.C. Chiles, and T.L. Rothstein: "Induction of CREB activity via the surface Ig receptor of B cells". *J. Immunol. 151*:880, 1993.). CREB/ATF proteins appear to regulate the expression of multiple genes through the CRE including immunologically important genes such as fos, jun B, Rb-1, IL-6, IL-1 (Tsukada, J., K. Saito, W.R. Waterman, A.C. Webb, and P.E. Auron: "Transcription factors NF-IL6 and CREB recognize a common essential site in the human prointerleukin 1β gene". *Mol. Cell. Biol. 14*:7285, 1994; Gray, G.D., O.M. Hernandez, D. Hebel, M. Root, J.M. Pow-Sang, and E. Wickstrom: "Antisense DNA inhibition of tumor growth induced by c-Ha-ras oncogene in nude mice". *Cancer Res. 53*:577, 1993), IFN-β (Du, W., and T. Maniatis: "An ATF/CREB binding site protein is required for virus induction of the human interferon B gene". *Proc. Natl. Acad. Sci. USA 89*:2150, 1992), TGF-β1 (Asiedu, C.K., L. Scott, R.K. Assoian, M. Ehrlich: "Binding of AP-1/CREB proteins and of MDBP to contiguous sites downstream of the human TGF-B1 gene". *Biochim. Biophys. Acta 1219*:55, 1994.), TGF-β2, class II MHC (Cox, P.M., and C.R. Goding: "An ATF/CREB binding motif is required for aberrant constitutive expression of the MHC class II DRa promoter and activation by SV40 T-antigen". *Nucl. Acids Res. 20*:4881, 1992.), E-selectin, GM-CSF, CD-8α, the germline Igα constant region gene, the TCR Vβ gene, and the proliferating cell nuclear antigen (Huang, D., P.M. Shipman-Appasamy, D.J. Orten, S.H. Hinrichs, and M.B. Prystowsky: "Promoter activity of the proliferating-cell nuclear antigen gene is associated with inducible CRE-binding proteins in interleukin 2-stimulated T lymphocytes". *Mol. Cell. Biol. 14*:4233, 1994.). In addition to activation through the cAMP pathway, CREB can also mediate transcriptional responses to changes in intracellular Ca^{++} concentration (Sheng, M., G. McFadden, and M.E. Greenberg: "Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB". *Neuron 4*:571, 1990).

The role of protein-protein interactions in transcriptional activation by CREB/ATF proteins appears to be extremely important. Activation of CREB through the cyclic AMP pathway requires protein kinase A (PKA), which phosphorylates $CREB^{341}$ on ser^{133} and allows it to bind to a recently cloned protein, CBP (Kwok, R.P.S., J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bachinger, R.G. Brennan, S.G.E. Roberts, M.R. Green, and R.H. Goodman: "Nuclear protein CBP is a coactivator for the transcription factor CREB". *Nature 370*:223, 1994; Arias, J., A.S. Alberts, P. Brindle, F.X. Claret, T. Smea, M. Karin, J. Feramisco, and M. Montminy: "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor". *Nature 370*:226, 1994.). CBP in turn interacts with the basal transcription factor TFIIB causing increased transcription. CREB also has been reported to interact with dTAFII 110, a TATA binding protein-associated factor whose binding may

Table 1: Oligonucleotide Stimulation of B Cells

ODN	Sequence (5' to 3')†	Stimulation Index' ^{3}H Uridine	Stimulation Index' IgM Production
1 (SEQ ID NO: 2)	GCTAGACGTTAGCGT	6.1 ± 0.8	17.9 ± 3.6
1a (SEQ. ID NO: 3)	.....T.....__.	1.2 ± 0.2	1.7 ± 0.5
1b (SEQ ID NO: 4)	.....Z.....__.	1.2 ± 0.1	1.8 ± 0.0
1c (SEQ ID NO: 5)	.....__....Z..	10.3 ± 4.4	9.5 ± 1.8
1d (SEQ ID NO: 6)	..AT..__..GAGC.	13.0 ± 2.3	18.3 ± 7.5
2 (SEQ ID NO: 7)	ATGGAAGGTCCAGCGTTCTC	2.9 ± 0.2	13.6 ± 2.0
2a (SEQ ID NO: 8)	..C_.CTC._G..__.....	7.7 ± 0.8	24.2 ± 3.2
2b (SEQ ID NO: 9)	..Z..CTC.ZG..Z.....	1.6 ± 0.5	2.8 ± 2.2
2c (SEQ ID NO: 10)	..Z..CTC._G..__.....	3.1 ± 0.6	7.3 ± 1.4
2d (SEQ ID NO: 11)	..C_.CTC._G..__..Z..	7.4 ± 1.4	27.7 ± 5.4
2e (SEQ ID NO: 12)	.....A__.....	5.6 ± 2.0	ND
3D (SEQ ID NO: 13)	GAGAACGCTGGACCTTCCAT	4.9 ± 0.5	19.9 ± 3.6
3Da (SEQ ID NO: 14)	.....__..C_.....	6.6 ± 1.5	33.9 ± 6.8
3Db (SEQ ID NO: 15)	.....__..C_.....G..	10.1 ± 2.8	25.4 ± 0.8
3Dc (SEQ ID NO: 16)	...C.A.....	1.0 ± 0.1	1.2 ± 0.5
3Dd (SEQ ID NO: 17)	.....Z.....	1.2 ± 0.2	1.0 ± 0.4
3De (SEQ ID NO: 18)	.....__.....Z.....	4.4 ± 1.2	18.8 ± 4.4
3Df (SEQ ID NO: 19)	.....__A.....	1.6 ± 0.1	7.7 ± 0.4
3Dg (SEQ ID NO: 20)	.....__..CC.G.ACTG..	6.1 ± 1.5	18.6 ± 1.5
3M (SEQ ID NO: 21)	TCCATGTCGGTCCTGATGCT	4.1 ± 0.2	23.2 ± 4.9
3Ma (SEQ ID NO: 22)	.....CT.....	0.9 ± 0.1	1.8 ± 0.5
3Mb (SEQ ID NO: 23)	.....Z.....	1.3 ± 0.3	1.5 ± 0.6
3Mc (SEQ ID NO: 24)	.....__..Z.....	5.4 ± 1.5	8.5 ± 2.6
3Md (SEQ ID NO: 25)	.....A__T.....	17.2 ± 9.4	ND
3Me (SEQ ID NO: 26)	.....__.....C..A.	3.6 ± 0.2	14.2 ± 5.2

Table 2: Cell Cycle Analysis with CpG ODN

Treatment	Percent of cells in G0	G1	SA+G2+M
Media	97.6	2.4	0.02
ODN 1a	95.2	4.8	0.04
ODN 1d	2.7	74.4	22.9
ODN 3Db	3.5	76.4	20.1
LPS (30 μg/ml)	17.3	70.5	12.2

The mitogenic effects of CpG ODN on human cells, were tested on peripheral blood mononuclear cells (PBMCs) obtained from two patients with chronic lymphocytic leukemia (CLL), as described in Example 1. Control ODN containing no CpG dinucleotide sequence showed no effect on the basal proliferation of 442 cpm and 874 cpm (proliferation measured by ^{3}H thymidine incorporation) of the human cells. However, a phosphorothioate modified CpG ODN 3Md (SEQ ID NO: 25) induced increased proliferation of 7210 and 86795 cpm respectively in the two patients at a concentration of just 1 μM. Since these cells had been frozen, they may have been less responsive to the oligos than fresh cells *in vivo*. In addition, cells from CLL patients typically are non-proliferating, which is why traditional chemotherapy is not effective.

Certain B cell lines such as WEHI-231 are induced to undergo growth arrest and/or apoptosis in response to crosslinking of their antigen receptor by anti-IgM (Jakway, J.P. et al., "Growth regulation of the B lymphoma cell line WEHI-231 by anti-immunoglobulin, lipopolysaccharide and other bacterial products" *J. Immunol. 137:* 2225 (1986); Tsubata, T., J. Wu and T. Honjo: B-cell apoptosis induced by antigen receptor crosslinking is blocked by a T-cell signal through CD40." *Nature 364*: 645 (1993)). WEHI-231 cells are rescued from this growth arrest by certain stimuli such as LPS and by the CD40 ligand. ODN containing the CpG motif were also found to protect WEHI-231 from anti-IgM induced growth arrest, indicating that accessory cell populations are not required for the effect.

To better understand the immune effects of unmethylated CpG ODN, the levels of cytokines and prostaglandins *in vitro* and *in vivo* were measured. Unlike LPS, CpG

Table 3: Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	% YAC-1 Specific Lysis* Effector: Target		% 2C11 Specific Lysis Effector: Target	
ODN	50:1	100:1	50:1	100:1
None	-1.1	-1.4	15.3	16.6
1	16.1	24.5	38.7	47.2
3Dd	17.1	27.0	37.0	40.0
non-CpG ODN	-1.6	-1.7	14.8	15.4

Neutralizing Activity of Methylated CpG Containing Oligos

B cell mitogenicity of ODN in which cytosines in CpG motifs or elsewhere were replaced by 5-methylcytosine were tested as described in Example 1. As shown in Table 1 above, ODN containing methylated CpG motifs were non-mitogenic (Table 1; ODN 1c, 2f, 3De, and 3Mc). However, methylation of cytosines other than in a CpG dinucleotide retained their stimulatory properties (Table 1, ODN 1d, 2d, 3Df, and 3Md).

Immunoinhibitory Activity of Oligos Containing a GCG Trinucleotide Sequence at or near both termini

In some cases, ODN containing CpG dinucleotides that are not in the stimulatory motif described above were found to block the stimulatory effect of other mitogenic CpG ODN. Specifically the addition of an atypical CpG motif consisting of a GCG near or at the 5' and/or 3' end of CpG ODN actually inhibited stimulation of proliferation by other CpG motifs. Methylation or substitution of the cytosine in a GCG motif reverses this effect. By itself, a GCG motif in an ODN has a modest mitogenic effect, though far lower than that seen with the preferred CpG motif.

Proposed Mechanisms of Action of Immunostimulatory, Neutralizing and Immunoinhibitory Oligonucleotides

Unlike antigens that trigger B cells through their surface Ig receptor, CpG-ODN did not induce any detectable Ca^{2+} flux, changes in protein tyrosine phosphorylation, or IP 3 generation. Flow cytometry with FITC-conjugated ODN with or without a CpG motif was performed as described in Zhao, Q et al.,(*Antisense Research and Development* 3:53-66